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ISOLATION OF DIHYDROFOLATE AND FOLYLPOLYGLUTAMATE SYNTHETASE ACTIVITIES FROM *NEUROSPORA*

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Abstract—The possible association of dihydrofolate synthetase (DHFS) and folylpolyglutamate synthetase (FPGS) in *Neurospora crassa* (FGSC 853, wild type) has been examined using mycelial extracts prepared and fractionated in the presence of protease inhibitors. DHFS and FPGS were assayed by following the incorporation of labelled glutamate into dihydrofolate and methylenetetrahydrofolate polyglutamate, respectively. Both of these activities were predominately cytosolic in mycelia that were harvested 24 hr after spore inoculation of defined minimal medium. Relatively small amounts of total mycelial DHFS activity were associated with mitochondrial fractions isolated by differential centrifugation. In contrast, *ca* 20% of the mycelial FPGS activity was mitochondrial. Treatment of the mitochondrial fractions with Triton X-100 suggested that these activities were not latent under the assay conditions employed. Separate peaks of DHFS and FPGS activity were observed when $(NH_4)_2SO_4$-fractionated protein was desalted and chromatographed on columns of either Mono Q HR, DEAE-cellulose, heparin agarose, Matrex Green A or Reactive Green 5. Gel filtration indicated average M_r values of 52 and 66×10^3 for DHFS and FPGS protein, respectively. Dihydrofolate synthetase protein was purified over 1000-fold by a protocol that included chromatography on DEAE-cellulose, DEAE-Sephacel, heparin agarose and Matrex Green A. The isolated protein lacked ability to glutamyl conjugate 5,10-methylene tetrahydrofolate. SDS–polyacrylamide gel electrophoresis of the Matrex Green A peak fractions revealed a major protein band of average M_r 52×10^3 whose concentration appeared to parallel DHFS activity. FPGS protein (average M_r 66×10^3), which lacked ability to glutamyl conjugate dihydropteroate, was recovered by a similar protocol. The reaction catalysed by DHFS protein displayed ATP dependency, was stabilized by glycerol, and product formation was favoured under alkaline conditions. The major catalytic properties of *Neurospora* DHFS are compared with those of other species.

INTRODUCTION

The one-carbon metabolism of prokaryotic and eukaryotic species centres on several folate-dependent reactions that have important roles in the biosynthesis of serine, glycine, methionine, formylmethionyl-tRNA, purines and thymidylate [2–5]. Folate-dependent enzymes generally display a preference for γ-glutamyl conjugated folate derivatives [6, 7] and these polyglutamates are commonly the principal forms of cellular folate [8–10]. The importance of these folylpolyglutamates in one-carbon metabolism is also supported by studies of mutant cell lines. In this connection, lesions which affect the expression of folylpolyglutamate synthetase (FPGS, tetrahydrofolate: L-glutamate gamma-ligase [ADP forming], EC 6.3.2.17) result in auxotrophies for products of one-carbon metabolism [11–15].

The glutamyl side chain of naturally occurring folates is formed by two distinct reactions catalysed by dihydrofolate synthetase (DHFS, EC 6.3.2.12) and FPGS, respectively (Eqns 1 and 2). Early studies of bacteria [16] and higher plants [17] showed that the DHFS reaction plays a vital role in organisms that generate folate *de novo*:

$$\text{Dihydropteroate} + \text{ATP} = H_2\text{PteGlu} + \text{ADP} + P_i \quad (1)$$

$$H_4\text{PteGlu} + n\text{ATP} + n\text{Glu} = H_4\text{PteGlu}_{n+1} + n\text{ADP} + nP_i. \quad (2)$$

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The abbrevations for folate derivatives are those suggested by the IUPAC-IUB Commission as summarized by Blakley and Benkovic [1].

Later work on *Escherichia coli* and *Corynebacterium* sp., showed that DHFS and FPGS are catalytic entities of a bifunctional protein, encoded by the *folC* gene [18–20]. However, it appears unlikely that such complexing occurs in higher plant tissues. In this regard, Ikeda and Iwai [17], and Imeson and Cossins [21] used distinct protocols for isolation of these two enzyme activities from pea seedlings. Thus, higher plant DHFS and FPGS may be distinct proteins that are encoded by separate genes. This possibility has not been verified to date.

Neurospora FPGS is encoded by the *met-6*$^+$ gene and has properties like those of the corresponding bacterial and mammalian synthetases [22, 23]. These latter studies did not examine the possible co-purification of DHFS and FPGS activity or determine whether purified *Neurospora* FPGS could generate dihydrofolate from dihydropteroate. As a result, it is not clear whether the DHFS and FPGS activities of this lower eukaryote occur as separate proteins, as appears to be the case in higher plants, or whether they occur as functional entities of the same proteins as in *E. coli* and *Corynebacterium*. The present work examines these possibilities.

RESULTS AND DISCUSSION

In preliminary work, we found that *Neurospora* DHFS, like the FPGS activity of this species [23], was very unstable and all activity was lost within 48 hr at 2°. Stability was however greatly improved when PMSF, benzamidine and 20% v/v glycerol were added to the extraction and isolation buffers. When mycelial extracts were prepared in isotonic buffer and then subjected to differential centrifugation (Table 1), both activities were principally associated with the cytosolic fraction. The crude mitochondrial pellet had only minor amounts of DHFS activity, a finding that is in contrast to earlier work on pea seedlings and spinach leaves [24]. On the other hand, *ca* 20% of the recovered FPGS was associated with the mitochondrial fraction. The mitochondrial DHFS and FPGS activities were not increased when the final suspending buffer contained 0.1% Triton X-100. This suggests that neither activity is latent under the conditions employed for isolation and assay. In wild type Chinese hamster ovary cells, slightly higher percentages of the total FPGS activity are associated with the mitochondria [25]. This localized activity clearly has importance in the accumulation of mitochondrial folate and the synthesis of glycine. The mitochondrial FPGS of *Neurospora* conceivably has a role in the generation of compartmented folates [26], but its significance in mitochondrial one-carbon metabolism remains to be elucidated.

When mycelial extracts in buffer A were fractionated with $(NH_4)_2SO_4$, the bulk of the DHFS and FPGS activity was associated with protein precipitating in the 45–70% range of saturation. This protein was desalted and examined by FPLC. Although both FPGS and DHFS coeluted from the cation exchange column (Fig. 1A), they were clearly separated by anion exchange chromatography (Fig. 1B). Larger scale separations of

Table 1. Intracellular localization of *Neurospora* FPGS and DHFS activities

Fraction	Enzyme activity* FPGS	DHFS	Ratio of FPGS/DHFS
Crude homogenate	175	5.7	31
Mitochondrial pellet	40.3	0.3	134
Cytosolic	167	6.0	28

*Glutamate incorporated (picokatals extract^{-1}) into 5,10-CH_2H_4PteGlu (FPGS) or H_2Pte (DHFS).

Mycelia were ground in sucrose buffered medium and the mitochondrial fraction was recovered by differential centrifugation (see Experimental).

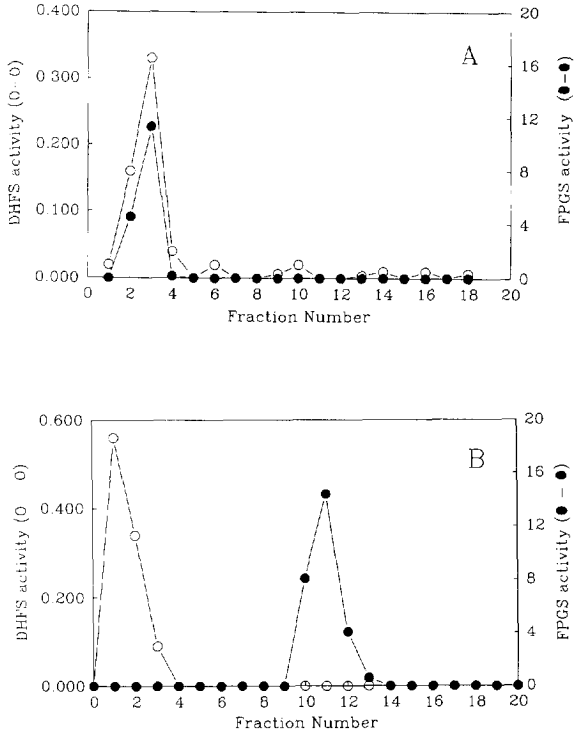

Fig. 1. Chromatography of *Neurospora* DHFS and FPGS activities. Samples (0.5 ml) of step 4 protein (Experimental) were applied to Mono S cation exchanger (A) and Mono Q HR 5/5 anion exchanger (B). The FPLC system (Pharmacia Liquid Chromatography System) was operated at room temperature, flow rate of 1 ml min^{-1} and with collection of 1 ml fractions. After washing for 5 min with buffer A containing 10% glycerol (Experimental), a linear gradient of buffer A containing 600 mM KCl was applied with maximal KCl concentration being reached after 25 min run time. DHFS (○) and FPGS (●) activities are expressed in picokatals ml^{-1}.

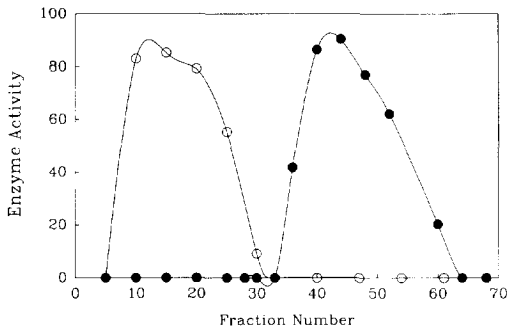

Fig. 2. Separation of DHFS and FPGS activities in extracts of *Neurospora* mycelia. Samples of desalted, step 3 protein were applied to 5 × 5 cm column of Whatman DE-52 cellulose pre-equilibrated in buffer A (Experimental). After washing (200 ml buffer A) to elute DHFS (○), FPGS (●) was eluted with a linear KCl gradient (Experimental). Enzyme activities are expressed in picokatals fraction^{-1}.

these activities were achieved on DEAE-cellulose (Fig. 2), a medium that also resolved the DHFS and FPGS activities of pea cotyledon extracts (data not shown). The conditions summarized in Fig. 2 were also employed in other separations. Thus, 1.5 × 10 cm columns of Matrex Green A, heparin agarose and Reactive Green 5 retained *Neurospora* FPGS protein which was subsequently eluted when the chloride gradient reached 0.6 M, 0.18 M and 0.6 M KCl in buffer A, respectively (data not shown). On the other hand, *Neurospora* DHFS protein in buffer A was retained by Matrex Green A and heparin agarose columns and then eluted when the gradient reached 0.2 M and 0.15 M KCl in buffer A, respectively. In contrast, DHFS protein in buffer A was not retained by the Reactive Green 5 column (data not shown). Thus, all of these media provided evidence for separate DHFS and FPGS proteins in *Neurospora*. Gel filtration using a calibrated column of Sephacryl S-200 gave apparent M_r values of 52 and 68 × 10^3 for the DHFS and FPGS proteins, respectively.

Protocols for isolation of more highly purified *Neurospora* DHFS and FPGS are summarized in Tables 2 and 3, respectively. The former enzyme was purified over 1000-fold with a yield of *ca* 12%. This step 7 protein did not form labelled polyglutamates when incubated with [^{3}H]-glutamate, ATP and either 5,10-CH_2H_4PteGlu or H_4PteGlu. SDS-polyacrylamide gel electrophoresis of DHFS-active fractions from the Matrex Green column

Table 2. Partial purification of *Neurospora* DHFS protein

Fractionation step	Volume (ml)	DHFS* (units ml^{-1})	Protein (μg m^{-1})	Spec. act. (units mg^{-1})	Purification (x-fold)	Yield (%)
1. Crude extract	800	1.8	3060	0.6	1.0	100
2. Streptomycin SO_4	820	1.6	2600	0.6	1.0	93
3. 45–70% $(NH_4)_2SO_4$	410	2.2	4300	0.5	0.8	62
4. DE-52 Cellulose	450	2.8	840	3.3	5.5	89
5. DEAE-Sephacel	100	5.4	530	10.2	17	38
6. Heparin agarose	60	5.9	120	49.2	82	25
7. Matrex Green A	15	11.3	18	628	1047	12

*DHFS activity is expressed in picokatals of glutamate incorporated under the standard reaction conditions (Experimental).

Table 3. Partial purification of *Neurospora* FPGS protein

Fractionation step	Volume (ml)	FPGS* (units ml^{-1})	Protein (μg ml^{-1})	Spec. act. (units mg^{-1})	Purification (x-fold)	Yield (%)
1. Crude extract	1050	1.6	12 700	0.12	1	100
2. Streptomycin SO_4	1100	1.5	4640	0.33	3	101
3. 45–70% $(NH_4)_2SO_4$	400	26.0	21 100	1.23	10	673
4. DE-52 Cellulose	320	8.3	1720	4.82	40	156
5. Reactive Green 5	225	8.8	20	440	3670	116
6. Heparin agarose	180	6.3	4	1580	13 100	66
7. Hydroxylapatite	43	9.3	7	1330	11 100	24
8. FPLC Mono Q HR 5/5	2	67.4	10	6740	56 200	12

*FPGS activity is expressed in picokatals of glutamate incorporated.

revealed a major protein band of *ca* 52×10^3 plus a minor band of lower M_r (data not shown). The intensity of silver staining of the major band was maximal in those fractions with the highest DHFS activity. The similarity between the apparent M_r after gel filtration and SDS–PAGE suggests that *Neurospora* DHFS, like FPGS [23], is a monomeric protein.

The protocol for isolation of *Neurospora* FPGS (Table 3) resulted in a 56 000-fold increase in specific activity and a final yield of 12%. Analysis of this protein by SDS–PAGE revealed a major protein of *ca* 66×10^3 accompanied by three minor, silver-staining bands (data not shown). Silver staining of the major protein band was greatest in fractions with the highest FPGS activity. These fractions had no detectable DHFS activity.

The properties of *Neurospora* DHFS were examined using step 7 protein. DHFS protein was relatively stable at 4° and retained almost 100% of its activity after 1 month of storage. The DHFS reaction had absolute requirements for ATP, pteroate, KCl, $MgCl_2$ and enzyme (Table 4). The rate of glutamate incorporation into the labelled reaction product was a linear function of time for at least 2 hr under the standard assay conditions. This tritiated product was readily cleaved by Zn–HCl treatment into a labelled compound that formed an azo dye derivative [27]. Acid cleavage of this dye derivative, followed by HPLC analysis [27] revealed a tritiated product with an average R_t of 10 min that co-chromatographed with authentic *p*-ABAGlu. Under these chromatographic conditions, the R_ts of authentic *p*-ABA, *p*-ABAGlu, *p*-$ABAGlu_2$ and *p*$ABAGlu_3$ were 6.2, 10, 25 and 32 min, respectively. However, we obtained no HPLC evidence for more highly glutamyl-conjugated products in any of the reaction systems examined. The DHFS reaction product, prior to acid cleavage, also supported the oxidation of NADPH in the presence of dihydrofolate reductase (data not shown). Based on these observations, we conclude that dihydrofolate is a major product of our *Neurospora* DHFS reaction system.

The pH optimum of DHFS activity was investigated using Tris buffers. In common with the synthetases of other species [17, 20, 28], activity was favoured under alkaline conditions and appeared to be maximal at pH 9.8. Activity was not detected when the reaction systems contained carbonate or Tris–glycine buffers. The apparent Michaelis constants for dihydropteroate, L-glutamate and ATP were 0.11, 290 and 50 μM, respectively. With the exception of dihydropteroate, these values are comparable to those reported earlier for the pea seedling enzyme [17, 28]. Enzyme activity was not affected by additions of pteroate, folic acid, H_4PteGlu or $H_4PteGlu_3$ (Table 5). However, H_2PteGlu and, to a lesser extent, its triglutamate inhibited the incorporation of ^{3}H-glutamate. In *E. coli*, inhibition of this reaction by H_2PteGlu has been interpreted as a possible control of folate synthesis [29]. Activity also appeared to be inhibited by relatively

Table 4. Requirements for glutamate incorporation by *Neurospora* DHFS

Deletions or additions to the standard reaction system	Activity (picokatals)
Complete*	0.69
−enzyme	n.d†
−ATP	n.d.
$-H_2$Pte	n.d.
−KCl	n.d.
+KCl (20 mM)	0.47
+KCl (30 mM)	0.70
$-MgCl_2$	n.d.
$+MgCl_2$ (5 mM)	0.50
$+MgCl_2$ (7.5 mM)	0.55
$+MgCl_2$ (10 mM)	0.70
$+MgCl_2$ (12 mM)	0.70
−BSA	0.55
+BSA (50 μg assay^{-1})	0.63
+BSA (100 μg assay^{-1})	0.69
−DMSO	0.58
+DMSO (10 μl reagent)	0.61
+DMSO (30 μl reagent)	0.69

*Standard reaction conditions were employed (see Experimental) with the exception that certain components were omitted or their concentrations were altered as indicated.

†n.d. = ^{3}H incorporation not significantly above background levels.

Step 7 protein (Table 2) was used as a source of DHFS activity.

Table 5. Effect of folates and adenosine phosphates on DHFS activity

Addition to reaction system	Final concentration (μM)	Relative activity (%)*
Pte	500	100
PteGlu	500	100
H_2PteGlu	10	64
	100	42
	500	34
$H_2PteGlu_3$	10	98
	100	68
	500	66
H_4PteGlu	500	100
$H_4PteGlu_3$	500	100
ADP†	1	115
	10	111
	50	73
AMP†	1	126
	10	122
	50	90

*Activity as a percentage of that shown by standard assay systems (see Experimental).

†ADP and AMP concentrations are mM.

high concentrations of ADP as noted earlier for the corresponding synthetases of *Corynebacterium* [19] and *Serratica indica* [30].

The separation of DHFS and FPGS activity when mycelial extracts were fractionated in the presence of protease inhibitors (Figs 1B, 2) suggests that these enzymes are not complexed as in *E. coli* [18]. A similar conclusion may be drawn from our observed chromatographic separation of pea cotyledon DHFS and FPGS activities. It is therefore conceivable that these proteins may be encoded by distinct genes. Thus, in *Neurospora*, lesions that affect the expression of FPGS activity will not necessarily impair the synthesis of monoglutamyl folates. It is noteworthy that mutation at the *met*-6 locus affects FPGS activity [14], but not the ability to incorporate *p*-ABA into simple, unconjugated folates [31]. Mycelia of the *met*-6 mutant also contain levels of DHFS activity that are comparable to those of the wild type [McDonald, D. and Cossins, E. A., unpublished data]. Recently, separate FPGS and DHFS genes have been isolated from *Saccharomyces* and the genes have been localized to separate chromosomes [32]. The reason for these fundamental differences in the structural organization of bacterial and fungal DHFS/FPGS activities is unclear. The production of distinct proteins to mediate the glutamylation of dihydropteroate or tetrahydrofolate may facilitate a transcriptional control of folate synthesis in fungi. These basic areas of folate biochemistry appear to warrant further investigation.

EXPERIMENTAL

Chemicals. PteGlu, pteroic acid, H_4PteGlu, heparin agarose, Reactive Green 5 and M_r marker proteins were obtained from Sigma. Polyglutamates of PteGlu and *p*-ABAGlu were from Dr B. Schircks Laboratories, Jona, Switzerland. [U-^{3}H]L-Glutamate from Amersham was diluted with carrier L-glutamate to give a final specific activity of 2.5 μCi 1.5 μmol^{-1}. H_4PteGlu$_n$ was generated from PteGlu$_n$ by catalytic hydrogenation [33] and dihydropteroate was synthesized by dithionite reduction of pteroic acid [34]. Folate concns were determined by ref. to extinction data [35]. Cellex D (OH form) and Biogel P6-DG were from Bio-Rad. Matrex Green A was purchased from Amicon. All other chemicals were from either Fisher Scientific or Sigma.

Organism and homogenate prepn. The Lindegren A wild type (FGSC 853) of *N. crassa* was cultured in Vogel's defined medium and freshly harvested conidiospores were used as inoculum [14]. Mycelia were harvested at 24 hr and homogenized (1:1.6, tissue wt to buffer A vol.) in 30 mM K Pi buffer (pH 7) containing 50 mM 2-mercaptoethanol, 1 mM PMSF, 1 mM benzamidine and 20% v/v glycerol (buffer A). The homogenate was passed through a continuous flow sonicator (Branson Sonic, Sonic Cell Disruptor 350) and centrifuged at 4500 *g* for 10 min. The resulting homogenate is designated step 1 protein. Seeds of pea (*Pisum sativum* L. cv Homesteader) were imbibed and germinated [21]. The cotyledons (100 g) of 1 and 6-day-old seedlings were homogenized in *ca* 200 ml of buffer A. The intracellular localization of *Neurospora* DHFS and FPGS activities was investigated by differential centrifugation of isotonic buffered extracts [36]. Frs from this procedure were examined for marker enzymes [37].

Sepn of DHFS and FPGS activities. Streptomycin sulphate (1% final concn) was added to the step 1 protein extract and nucleic acids were removed by centrifugation (6500 *g* for 10 min) to give step 2 protein. Fractionation of protein with $(NH_4)_2SO_4$ was as previously described [23] and that precipitating in the 45–70% range of satn was redissolved in buffer A (step 3 protein). After desalting by passage through BioGel P6-DG in buffer A (step 4 protein) the extract was applied to a 5 × 5 cm column of DE-52 cellulose pre-equilibrated in buffer A. The column was washed with 200 ml buffer A to elute DHFS activity, followed by a linear gradient of 200 ml buffer A to 200 ml buffer A containing 100 mM KCl to recover FPGS protein. Frs of 10 ml were collected. Sepn of these enzymes activities was also achieved by FPLC of step 4 protein as summarized in Fig. 1.

Purification of DHFS activity. Samples (*ca* 200 ml) of step 4 protein were applied to a 5 × 5 cm column of DEAE-Sephacel that had been pre-equilibrated in 30 mM Tris buffer (pH 9.0), containing 100 mM mercaptoethanol, 20% glycerol, 1 mM PMSF and 1 mM benzamidine. A linear KCl gradient (0–0.75 M in this buffer) was applied and frs of 10 ml were collected. DHFS activity, associated with frs 20–32, was pooled (step 5 protein) and concd by ultrafiltration (Amicon Stirred Cell, 10 μm filter). After desalting with BioGel P6-DG, protein was applied to a 2.5 × 10 cm column of heparin agarose that had been pre-equilibrated with buffer A (pH 6). Frs of 6 ml were collected. DHFS activity (frs 40–50) was eluted by applying a linear KCl gradient (0–0.5 M in buffer A at pH 6). This step 6 protein was pooled, concd as above, applied to a 2.5 × 10 cm column of Matrex Green A in buffer A (pH 8) and subsequently eluted with a linear KCl gradient (0–1 M) in buffer A to give step 7 protein.

Purification of FPGS activity. Step 4 protein, isolated as described above, was chromatographed on a 1.5 × 15 cm column of Reactive Green 5 pre-equilibrated with buffer A. Frs of 6 ml were collected. FPGS protein was eluted (frs 42–58) by applying a KCl gradient (0–0.8 M) in buffer A. This step 5 protein was applied to heparin agarose as in the DHFS isolation (see above) but eluted at pH 7. The resulting step 6 protein was concd, desalted and applied to a 1.5 × 5 cm column of hydroxylapatite pre-equilibrated in 10 mM buffer A. Under these conditions, FPGS protein (step 7) passed through the column and was applied to a Mono Q HR 5/5 FPLC column pre-equilibrated with buffer A containing 10% v/v glycerol. A KCl gradient (0.01–0.6 M) in this buffer was used to elute step 8 FPGS protein.

Assay of DHFS and FPGS activities. The method of ref. [19] was modified for DHFS assay. Reaction systems

(1 ml) contained: Tris–HCl (pH 10.3) 300 μmol, ATP 4 μmol, dihydropteroate 5 nmol, BSA 0.1 mg, DMSO 30 μl reagent, KCl 30 μmol, $MgCl_2$ 10 μmol, L-glutamate (2.5 μCi of ^3H) 1.5 μmol, 200 μl enzyme. After incubation at 37° for 2 hr the tubes were transferred to an ice bath and 3 ml of 30 mM 2-mercaptoethanol was added. In the FPGS assay, modified after ref. [38], 1.15 ml reaction systems contained: Tris–HCl, ATP, glutamate, KCl, $MgCl_2$ and enzyme as for the DHFS assay, but H_4PteGlu 100 nmol and HCHO 1 μmol replaced dihydropteroate. Under these standard assay conditions, all substrates except L-glutamate were close to saturating. Activities are expressed in terms of glutamate incorporated (katals) into H_2Pte (DHFS) and 5,10-CH_2H_4PteGlu (FPGS). In kinetic studies, linear Lineweaver–Burk plots were obtained when the reaction systems, containing varying concns of dihydropteroate (5,10-CH_2H_4PteGlu) or ATP, were incubated for 1 hr with fixed concns of the other substrates. The labelled products of both reactions were isolated using DE-52 cellulose [39] and characterized by HPLC after cleavage to *p*-ABAGlu derivatives [27]. Dihydrofolate was also identified by use of Sigma dihydrofolate reductase [40]. Protein was assayed by the method of ref. [41] and SDS–PAGE according to ref. [42].

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